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Comparison of Flavonoid Composition of Red Raspberries (*Rubus idaeus* L.) Grown in the Southern United States

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Supporting Information

ABSTRACT: Raspberry flavonoid compounds have significant antioxidant activities, and regular consumption may help prevent and/or moderate chronic diseases. Targeted metabolite profiling is useful to identify compounds contributing to these antioxidant properties and health benefits and for tailored breeding for functional foods. In this study, metabolomic variation was determined among three fall-fruiting red raspberry cultivars ('Autumn Britten', 'Caroline', 'Nantahala') grown at three North Carolina locations differing in elevation and average day/night temperatures. 'Nantahala' was specifically bred for the mountainous regions of the southern United States. Ten flavonoid compounds were detected by liquid chromatography−time-of-flight−mass spectrometry (LC-TOF-MS). Of those, cyanidin-3-glucoside, cyanidin-3-sophoroside, cyanidin-3-rutinoside, cyanidin-3-sambubioside, and quercetin-3-glucoside were quantified against external standards. Variation in flavonoid composition was primarily attributed to genotype and associated with night temperature and hours exposed to temperatures over 29 °C. 'Nantahala' had particularly high levels of cyanidin-3-sambubioside, indicative of its purple raspberry lineage. Quercetin-3-glucoside levels increased the most with elevated temperatures.

KEYWORDS: *raspberries, Rubus idaeus, anthocyanins, antioxidant capacity, FRAP, LC-MS, LC-TOF-MS, phenolics, flavonoids, PCA, targeted metabolite profiling*

INTRODUCTION

The international and domestic market for raspberries has grown due in part to consumer interest in more dietary intake of fruits and vegetables for nutrition and health.[1,2] Red raspberries in particular have a high fresh and processed market value.[3,4] Red raspberries contain a range of vitamins, minerals, and phytochemicals that are essential for health and are related to reduced disease risk.[2,5] These phytochemicals can function as antioxidants that slow or stop damage to cellular DNA, proteins, and lipids caused by reactive oxygen species (ROS).[6] The major antioxidants found in red raspberry are anthocyanins and ellagitannins, which compose up to 85% of total phenolics and 50% of total antioxidant power.[3,7,8] Numerous in vitro trials with raspberry have demonstrated effectiveness for disease prevention and anti-inflammation, antibiotic, and anticancer activities.[3,9]

Metabolomics is a field of study that has emerged as an important tool for comparative, detailed phenotyping in many organisms.[10] In the past, phenotype, which includes measurable physical, chemical, and molecular characteristics, was observable only in outwardly visible traits. However, with the advancements of analytical technology, including metabolomics, the observable phenotype has been expanded to include quantitative measurements at the molecular level.[11] Metabolomics technologies have progressed such that metabolites can now be detected accurately and continuously by retention time and exact mass.[12] Target compound analysis is a metabolomics technique that measures only specific metabolites or groups of metabolites and is often employed in the plant sciences.[10] One of the earliest metabolomics-type studies on red raspberry was Mullen et al.'s use of LC-MS targeted analysis to characterize eight anthocyanin compounds in 'Glen Ample' that had been previously misidentified using HPLC alone.[13] Because variation in antioxidant compounds and human essential nutrients is evident at the metabolite level, these related profiling techniques could be especially useful to measure variation among a population or species to develop nutritionally enhanced cultivars.[14] Employing metabolomics and marker-assisted breeding strategies in raspberry provides an opportunity to improve the nutritional value and content of bioactive compounds.[2,5]

Most compositional studies on raspberry have been done with fruit grown in temperate climates with cool production seasons.[2,7,13,15−21] Most red raspberry accessions are not acclimated to grow in warm climates, where high summer temperatures can lead to heat stress and mild winters (>10 °C)

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Table 1. Locations of Replicated Trials Where Fruit Samples of Each Cultivar Were Harvested[a]

location	latitude, longitude	elevation (m)	av daily high temp (°C)	av night temp (°C)
1. Piedmont Research Station, Salisbury, NC	35.697 N, −80.622 W	214	32	21
2. Mountain Horticultural Crops Research Station, Fletcher, NC	35.427 N, −82.559 W	630	28	16
3. Upper Mountain Research Station, Laurel Springs, NC	36.402 N, −81.297 W	917	24	14

[a]Each location varies in elevation and growing season daily maximum and minimum temperatures from July 15 to September 20, 2010.

Table 2. Concentrations of Flavonoid Compounds in Three Raspberry Cultivars Quantified by LC-TOF-MS and Comparison to External Standards[a]

	concentration (mg/g DW ± standard error)		
compound	Autumn Britten	Caroline	Nantahala
cyanidin-3-glucoside	2.510 ± 0.149 b	5.039 ± 0.305 a	2.552 ± 0.071 b
cyanidin-3-rutinoside	0.132 ± 0.016 a	0.044 ± 0.004 b	0.036 ± 0.015 b
cyanidin-3-sambubioside	2.763 ± 0.884 b	0.832 ± 0.124 b	8.909 ± 1.131 a
cyanidin-3-sophoroside	1.716 ± 0.143 c	5.878 ± 0.450 a	2.622 ± 0.213 b
quercetin-3-glucoside	0.031 ± 0.006 a	0.028 ± 0.005 ab	0.014 ± 0.002 b

[a]Data values for each cultivar are the mean of 18 samples, averaged across harvest locations. Concentrations within the same row labeled with the same letter are not significantly different ($p < 0.05$).

can interfere with chilling hour accumulation. The optimum temperatures for red raspberry growth are 18−21 °C (air)/24−27 °C (soil). Above these temperatures, photosynthesis shuts down and plant and fruit size are reduced.[22,23] Heat stress is evidenced by smaller berry size, lower yields, and poor fruit quality of red raspberries where temperatures may exceed 32 °C during the summer fruiting season. Higher than ideal temperatures have been shown to decrease anthocyanin content in several fruit species.[1] In contrast, a study on 'Glen Ample' raspberries found that as postflowering temperature increased from 12 to 24 °C, total phenolics, total anthocyanins, and antioxidant capacity significantly increased; however, these findings were partially attributed to decreasing fresh weight with increasing temperature.[21] Effects of daily temperature ranging from 20 to 25 °C in the field on raspberry fruit anthocyanins and phenolics are not known.

In this study, we used LC-TOF-MS-based targeted analysis to detect and measure flavonoid compounds in three primocane-fruiting cultivars of red raspberry grown under polytunnel cultivation in central and western North Carolina. On the basis of previous research, [6,15,16] we expected to see differences among cultivars and locations. By assembling a metabolite profile of flavonoid compounds in raspberry, we expect to track the warm climate effects on raspberry antioxidants and flavonoids and simultaneously decipher genotype × environment variation.

MATERIALS AND METHODS

Plant Materials. Primocane-fruiting red raspberry cultivars 'Autumn Britten', 'Caroline', and 'Nantahala' were harvested from July 22 to September 20, 2010, from three research stations located in North Carolina with various elevations and temperature fluctuations (see Table 1). Standard practices for raspberry cultivation in North Carolina were followed.[22] Fruit was grown in replicated trials under quonset-style rounded top high tunnels covered in polyethylene greenhouse-grade plastic at each location. Fruit samples were frozen immediately after harvest for 24 h at −20 °C and then stored at −80 °C until lyophilization. Freeze-dried samples were stored at −20 °C until used.

Standards. Cyanidin-3-sambubioside, cyanidin-3-rutinoside, and cyanidin-3-sophoroside were obtained from Polyphenols Laboratories AS (Sandnes, Norway). Cyanidin-3-glucoside and pelargonidin were purchased from Extrasynthese (Genay, France). Quercetin-3-glucoside was obtained from Sigma-Aldrich (St. Louis, MO).

Sample Preparation. For each sample, 20 g of freeze-dried raspberries was homogenized with a mortar and pestle, and seeds were separated from raspberry powder through a 2 mm mesh sieve. Two extractions were performed, where solvent containing 5 mL of LC-MS grade methanol in water (60:39) with 1% formic acid was added to 100 mg of powder and vortexed for 1 min to mix. Samples were centrifuged at 4 °C for 20 min at 2790*g*. Supernatant from each extraction was filtered through Whatman no. 1 paper, pooled, and stored at −80 °C in 15 mL brown glass tubes until analysis.

HPLC Analysis Conditions. Prepared extracts were filtered through 0.2 μm PTFE membranes, and 50 μL of each sample was diluted in 450 μL of LC-MS grade methanol to a concentration of 1 μg/mL. Stock solutions for standard curve calculation were prepared from 0.005−10 μg/mL for each standard. Five microliter aliquot samples were injected at ambient temperature into an Agilent 1200 series HPLC system equipped with a binary solvent delivery manager and a sample manager (Agilent Corp., Santa Clara, CA) and fitted with an Agilent ZoRbax Eclipse XDB-C_{18} (4.6 × 150 mm, 5 μm particle size) chromatography column. The column temperature was maintained at 30 °C with a flow rate of 0.4 mL/min. The mobile phases consisted of 2% formic acid in water for A and 2% formic acid in acetonitrile for B, with the following elution gradient: 0−2 min, 2% B; 2−15 min, 2−20% B; 15−30 min, 20−45% B; 30−50 min, 45−98% B; 50−60 min, 98% B; 60−70 min, 2% B. Each sample was run in duplicate, with means averaged.

TOF-MS Conditions. Mass spectra analysis was performed on an Agilent 6220 MSD/TOF mass spectrometer equipped with a dual-spray electrospray ionization (ESI) source (Agilent Corp.). Data were collected from both positive and negative ESI modes, scanning a m/z 50−1000 range. In positive ion mode capillary voltage was set at 3500 V, nebulizer pressure at 45 psi, drying gas temperature at 325 °C, and drying gas flow at 11 L/min. The same conditions were used in negative ion mode, with capillary voltage decreased to 3000 V. Raw data were processed using the Agilent MassHunter Qualitative Analysis software (Agilent Corp.), and compound identification was done on the basis of mass spectra, retention time compared to authentic external standards, accurate mass data, and previously reported findings.[2,6,13,21] For those compounds for which standards

were available, concentration was reported as mg/g dry weight calculated from the prepared standard curve (Table 2; see also the Supporting Information). Mass spectra and retention time are reported for all identified compounds (Table 3).

Table 3. Mass Spectral Data of Detected Compounds Using LC-TOF-MS

compound	formula	t_R (min)	$M^{\pm}$ (m/z)
cyanidin-3-glucoside	$C_{21}H_{20}O_{11}$	24.5	447.09^-
cyanidin-3-(2^G-glucosyl-rutinoside)	$C_{33}H_{40}O_{20}$	24.0	755.18^-
cyanidin-3-rutinoside	$C_{27}H_{30}O_{15}$	24.6	593.15^-
cyanidin-3-sambubioside	$C_{26}H_{28}O_{15}$	24.1	579.14^-
cyanidin-3-sophoroside	$C_{27}H_{30}O_{16}$	23.6	609.15^-
kaempferol-3-glucuronide	$C_{21}H_{18}O_{12}$	26.4	461.16^-
pelargonidin-3-glucoside	$C_{21}H_{20}O_{10}$	26.0	433.03^+
pelargonidin-3-rutinoside	$C_{27}H_{30}O_{14}$	26.3	579.06^+
pelargonidin-3-sophoroside	$C_{27}H_{30}O_{15}$	25.2	596.06^+
quercetin-3-glucoside	$C_{21}H_{20}O_{12}$	29.0	463.09^-

Measurement of Total Anthocyanins Content. Total anthocyanins of the prepared extracts were determined by the pH-differential method described by Giusti and Wrolstead[24] using a Shimadzu UV-2450 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) set to read absorbance at 510 and 700 nm, through 1 cm path length disposable cuvettes. Samples were replicated and analyzed in triplicate, with means calculated. Total anthocyanins were reported as milligrams of cyanidin-3-glucoside equivalents per liter.

Total Phenolics Measurement. Total phenolics of the prepared extracts were determined by the Folin−Ciocalteu method described by Singleton and Rossi.[25] Absorbance of samples and gallic acid standards was read at 765 nm by spectrophotometer (Shimadzu UV-2450, Shimadzu Scientific Instruments), and samples were analyzed in triplicate, with means calculated. Total phenolics were reported as milligrams of gallic acid equivalents (GAE) per 100 g of fresh weight.

Antioxidant Capacity Measurement. The antioxidant capacity of the prepared extracts was determined by the ferric reducing antioxidant power (FRAP) assay initially described by Benzie and Strain.[26] Absorbance of samples and Trolox standards was read at 593 nm by spectrophotometer (Shimadzu UV-2450, Shimadzu Scientific Instruments), and samples were replicated and analyzed in triplicate, with means calculated. Antioxidant capacity was reported as micromoles of Trolox equivalents per gram of fresh weight.

Statistical Analysis. SAS (SAS Institute, Cary, NC) and SIMCA P+ 12.0 (Umetrics, Umeå, Sweden) statistical software programs were used to perform statistical analyses. The experimental design was 3 × 3 factorial (3 cultivars, 3 locations). One-way analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were used to evaluate differences in mass abundance of detected compounds and to examine cultivar, location, time, and temperature effects on the concentration of each quantified and detected metabolite and total phenolics, anthocyanins, and antioxidant capacity. LSMEANS with a Bonferroni correction was used to make pairwise comparisons among sample groups (cultivars, locations, harvest dates, average day/night temperatures). Multivariate data sets collected from LC-TOF-MS were analyzed using principal component analysis (PCA). Using a multidimensional vector approach and eigen analysis linear algebra, PCA determines those basic eigenvectors that most contribute to total variance. The eigenvectors are calculated through linear combinations of the standardized variables, and the eigenvector that correlates with the largest eigenvalue has the same direction as the first principal component, and so on with the remaining principal components. When the samples are plotted in a two- or three-dimensional space over the main principal components, those contributing the most to total variance provide the best sample separation.[10,11,17]

RESULTS AND DISCUSSION

Using LC-TOF-MS, 10 flavonoid compounds were detected in the fruit samples (Table 3; Figure 1), and 5 of those were

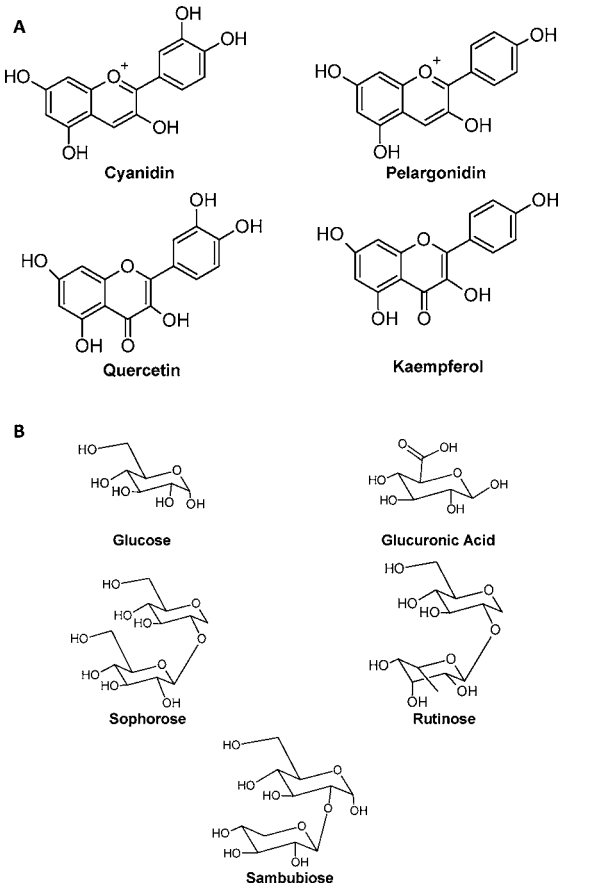

Figure 1. (A) Aglycones of detected flavonoid compounds in red raspberry samples. Cyanidin and pelargonidin are the primary anthocyanin compounds, and quercetin and kaempferol are flavonol compounds found in smaller quantities. (B) Glycosides detected of the identified compounds (Table 3), normally found attached to the hydroxyl group on the third or fifth carbon of the flavonoid aglycone.

quantified using external standards (Table 2). The majority of compounds were detected in >90% of the samples, whereas cyanidin-3-(2^G-glucosylrutinoside) was detected in 72% of the samples and pelargonidin-3-rutinoside was detected in 52% of the samples. Anthocyanin accumulation in red raspberry has been shown in previous research to be mainly under genetic control, with some environmental modifiers present.[1,2,15,16] Our study indicates the same effect, as the flavonoid profiles were not dramatically different among the three harvest locations, and significant temperature and location effects were found in only a few compounds.

Pelargonidin-3-glucoside, pelargonidin-3-sophoroside, and pelargonidin-3-rutinoside were detected using accurate mass and retention time data (Table 3) and by comparison to previous studies.[2,13] Examination of ion abundance versus m/z data indicated no significant variation among cultivars for pelargonidin-3-glucoside. 'Autumn Britten' is particularly abundant in pelargonidin-3-sophoroside, and 'Autumn Britten' and 'Nantahala' had similarly higher amounts of pelargonidin-3-rutinoside in comparison to 'Caroline' (Figure 2). Pelargonidin is the last anthocyanin to accumulate during ripening[3] and may

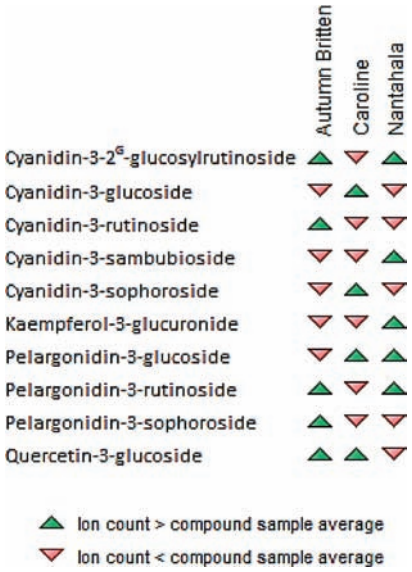

Figure 2. Flavonoid compounds detected by LC-TOF-MS analysis. Ion counts for each compound were averaged across all samples, and averages for each cultivar are reported as above average or below average, indicating approximate genotypical variation.

be affected by general fruit load and partitioning of resources. Freeman et al.[15] found that phenolic levels in red raspberry were highest at the beginning and end of the harvest season.

For each of the cyanidin glycosides measured, significant differences among flavonoid composition were attributed to genotype, with some covariate effects due to temperature changes and location. Cyanidin-3-glucoside and cyanidin-3-sophoroside, generally characterized as important red raspberry anthocyanins,[16] were the most abundant flavonoids quantified ($p < 0.0001$) in 'Caroline' (Table 2; Figure 2), suggesting that 'Caroline' contained higher concentrations of glucose and/or glucose-associated enzymes. A single glucose molecule attached to a flavonoid aglycone creates a glucoside, and two bonded glucose molecules form the sophorose glycoside.[23,31] For 'Caroline', a significant positive linear correlation ($p = 0.048$) was established between cyanidin-3-glucoside concentration and average hours of exposure to temperatures over 29 °C during the harvest period (~7 days prior to harvest date). Additionally, a significant inverse linear correlation ($p = 0.023$) was established between cyanidin-3-sophoroside concentration and average night temperature and between cyanidin-3-sophoroside concentration and average hours of exposure to temperatures over 29 °C ($p = 0.043$), which is consistent with other studies showing significant decreases in cyanidin-3-sophoroside with increasing temperatures.[2,21] It also may be that cooler night temperatures are important for the accumulation of some anthocyanins, but not for others. The contrasting temperature relationships seen between cyanidin-3-glucoside and cyanidin-3-sophoroside in 'Caroline' suggest that high temperatures may have some negative impact on the enzymes responsible for the formation of sophorose or its attachment to the cyanidin aglycone.

Cyanidin-3-rutinoside was significantly higher ($p < 0.0001$) in 'Autumn Britten' than in 'Caroline' or 'Nantahala' (Table 2; Figure 2). de Ancos et al. found that 'Autumn Bliss', which has the same parentage as 'Autumn Britten', had high levels of cyanidin-3-rutinoside.[27] Higher temperatures throughout the harvest season also seem to enhance the production of cyanidin-3-rutinoside, as a significant interaction between cultivar and location was present ($p = 0.0004$), with the highest levels found in 'Autumn Britten' samples from locations 1 and 2 (Table 1). Minor variations among concentrations of cyanidin-3-glucoside, cyanidin-3-sophoroside, and cyandin-3-rutinoside may also be due to slight differences in harvest maturity, as these three compounds are found to increase throughout ripening.[6]

Cyanidin-3-sambubioside was found in significantly higher concentrations ($p = 0.0005$) in 'Nantahala' versus the other two genotypes. Cyanidin-3-sambubioside is typically found in higher levels in black and purple raspberries,[23] and this correlates with the breeding background of 'Nantahala'. The cultivar is one-fourth 'Royalty',[28,29] a backcross between a hybrid purple and a red raspberry.[30] Both 'Autumn Britten' and 'Nantahala' clearly show that "knowledge of a genotype's anthocyanin sugars frequently provides evidence of its probable ancestry".[31] Knowing the distinct anthocyanin profiles associated with different genotypes of red raspberry can be important for authentication of botanical products or to help in screening wild and domesticated germplasm bases for related genotypes and species with similar or enhanced health benefits and antioxidant power.[1]

The flavonols quercetin-3-glucoside and kaempferol-3-glucuronide were detected in each analyzed sample (Table 3; Figure 2). By comparing ion counts between the two compounds, it was determined that quercetin-3-glucoside is more abundant than kaempferol-3-glucuronide, consistent with the findings of a previous study of 'Glen Ample' red raspberries.[32] Both quercetin and kaempferol decrease during ripening,[6] consistent with our fully ripened fruit samples. A significant genotypic effect ($p = 0.013$) was found for quercetin-3-glucoside, quantified using an external standard (Table 2). 'Autumn Britten' had higher levels of quercetin-3-glucoside than 'Nantahala'. In contrast, no significant differences were found among cultivars for the relative abundance of kaempferol-3-glucuronide. In addition to genotypic variation, quercetin levels are particularly sensitive to environmental variations such as light intensity, temperature, and soil conditions.[16] In our study, the significant variation in quercetin-3-glucoside levels is attributed to location and temperature effects among the measured samples.

A significant interaction between the effects of cultivar and location ($p = 0.015$) is present for quercetin-3-glucoside, with the highest levels seen in 'Autumn Britten' and 'Caroline' grown at locations 1 and 2 (Table 1). Higher levels of quercetin-3-glucoside at these two warmer locations are further explained by significant covariate relationships between cultivar and hours of exposure over 29 °C during the harvest period ($p = 0.014$) and between cultivar and average night temperatures ($p = 0.03$). For each genotype evaluated, as night temperatures increased from 14.2 to 25 °C and time above optimal growth conditions during the harvest period increased from 0 to 7.6 h, levels of quercetin-3-glucoside also increased.

In addition to employing univariate statistical tools to measure cultivar and location effects on individual flavonoid compounds, PCA was utilized to analyze variation among the samples based on the constructed flavonoid profiles as a whole. In this instance, the samples clearly separated into three groups corresponding to the three genotypes involved (Figure 3A), verifying that flavonoid composition is a distinguishing characteristic among cultivars.[1] The first principal component, plotted on the *x*-axis, explains 79% of total sample variance and

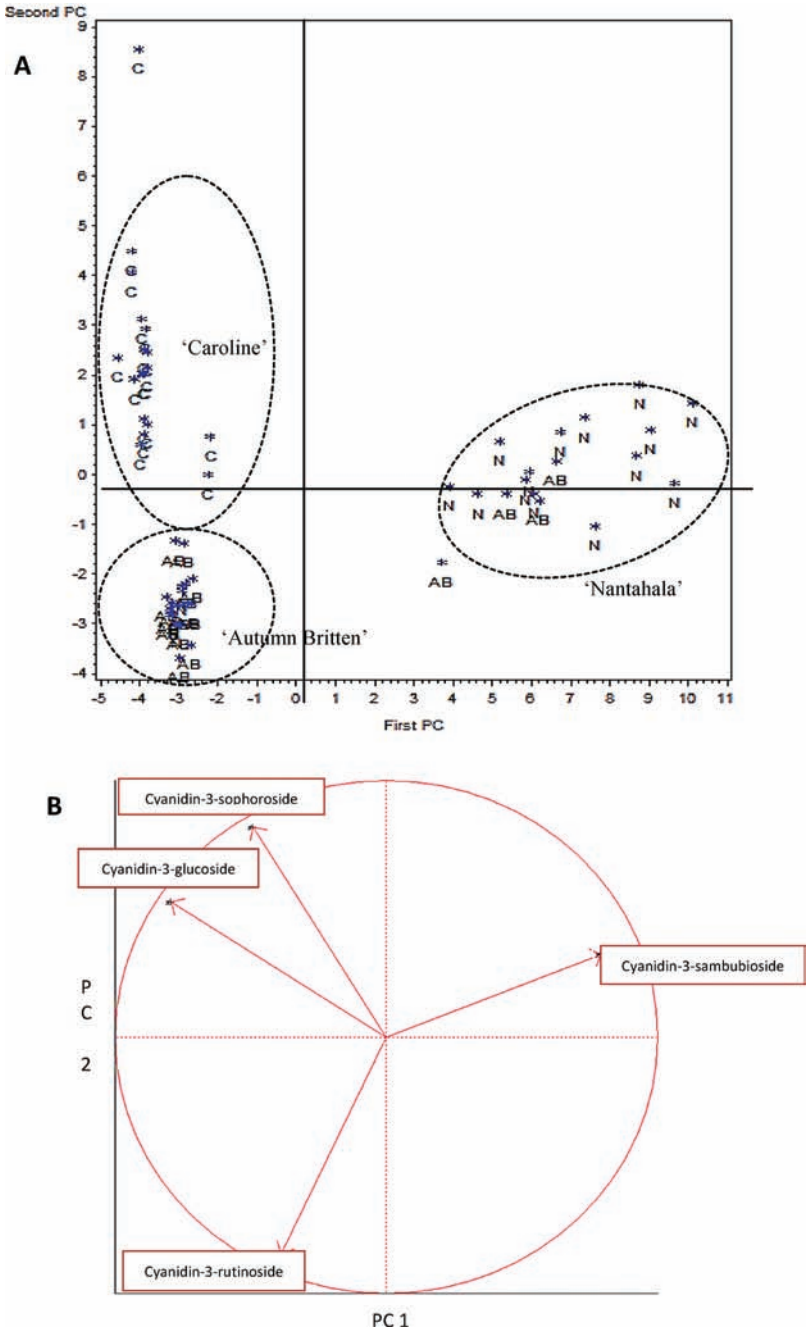

Figure 3. (A) PCA scores plot of samples characterized by LC-TOF-MS. Each point represents a sample, with 18 samples per cultivar measured. Clustering of the samples by genotype shows significant genotypical control of flavonoid composition. (B) PCA loading plot corresponding wth the scores plot in panel A. Compounds detected by external standards are mapped and used to create a multivariate data set and metabolite profile of the raspberry samples.

is associated with concentrations of cyanidin-3-sambubioside. The second principal component, plotted on the *y*-axis, explains 18% of variance and is designated by the difference in concentrations of cyanidin-3-sophoroside/glucoside and cyanidin-3-rutinoside. The corresponding loading plot (Figure 3B) shows the primary compounds responsible for the separation, which also correspond with the characteristic compounds defined for each cultivar above using ANOVA. 'Autumn Britten' falls in the third quadrant of the scores plot in Figure 3A and corresponds to cyanidin-3-rutinoside in Figure 3B. Cyanidin-3-rutinoside was found to be significantly higher in 'Autumn Britten' compared to the other two cultivars. The same is true of 'Caroline' and cyanidin-3-sophoroside in the fourth quadrant and 'Nantahala' and cyanidin-3-sambubioside in the first and second quadrants. PCA gave similar results in a previous study,[18] where clear separation occurred among progeny of a 'Glen Moy' × 'Latham' cross based on concentrations of cyanidin-3-sophoroside and cyanidin-3-rutinoside. These types of results show that PCA can be useful in breeding for functional foods, by allowing for quick selection and screening for individuals with high concentrations of phytochemicals of interest.[18]

Assays measuring total anthocyanins (Figure 4A), total phenolics (Figure 4B), and antioxidant capacity (Figure 4C)

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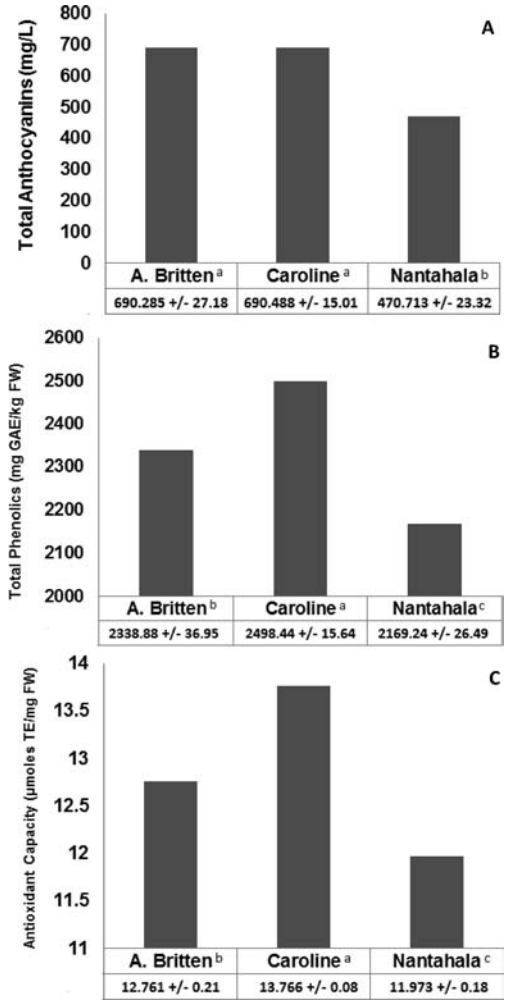

Figure 4. (A) Total anthocyanins, (B) total phenolics, and (C) antioxidant capacity averaged by cultivar. For each respective graph, those cultivars labeled with the same lower case letter are not significantly different.

showed significant cultivar effects ($p < 0.0001$), with some interacting location effects ($p = 0.0001$). 'Autumn Britten' and 'Caroline' had significantly higher total anthocyanin values than 'Nantahala', and the highest measurements were seen in samples from location 2. Total phenolics and antioxidant capacity assays showed similar significance patterns, with the highest values for 'Caroline', followed by 'Autumn Britten' and 'Nantahala'. Total phenolics and antioxidant capacity went up with increasing harvest season temperatures, as the highest measurements were seen in 'Caroline' and 'Autumn Britten' samples from the two warmest locations at locations 1 and 2. These measurements positively correlate with the increases in cyanidin-3-glucoside, cyanidin-3-rutinoside, and quercetin-3-glucoside seen with increased day and night temperatures at different locations. In a similar study, total anthocyanins, total phenolics, and FRAP, measured on a fresh weight basis, increased significantly as postflowering temperature increased from 12 to 24 °C in 'Glen Ample' red raspberries grown in a climate-controlled phytotron.[21] Our study shows that temperature may affect phytochemical accumulation throughout the entire harvest season, in addition to during fruit development. Furthermore, these effects may become more pronounced at above-optimal production temperatures (>27 °C). Some concentrating effects may be seen as berry weight decreases and temperature increases;[21] however, in our study measurements were taken from lyophilized samples. With interest in breeding for increased health-beneficial properties and the possible need for adaptation to temperature extremes associated with climate change, it is important to understand how temperature affects phytochemical content and antioxidant power, so functionally enhanced fruits can be developed without compromising fruit quality or yield.

Significant correlations ($p < 0.0001$) were found between total phenolics and total anthocyanins ($R^2 = 0.544$) and between total anthocyanins and antioxidant capacity ($R^2 = 0.596$), indicative of the contribution of anthocyanin compounds to total phenolic composition and overall antioxidant reducing power of the samples (Figure 5A,B). Additionally, a strong correlation ($p < 0.0001$) was found between total phenolics and antioxidant capacity ($R^2 = 0.912$), showing the combined contribution of anthocyanins and other phenolics to the antioxidant reducing ability of the samples (Figure 5C). Similar correlations have been found in other studies with raspberries,[7,15,17,18] showing the consistent ability of raspberry fruits of different genetic profiles and production environments to deliver health-beneficial antioxidant activity in vitro and in vivo.

An additional factor that must be considered in this study is the use of polytunnel cultivation. High tunnels have proven to be a great advantage to the raspberry industry through harvest season extension, with increased yields and improved fruit quality.[33,34] Red raspberries grown in North Carolina under high tunnels show better fruit quality and higher yields because the fruit are kept dry, causing fewer incidences of fungal pathogens (Fernandez, unpublished data). Conditions within the high tunnel are similar to the field, but with reduced light exposure and slightly warmer temperatures. Kassim et al.[2] found that levels of cyanidin-3-sophoroside, cyanidin-3-glucoside, and pelargonidin-3-rutinoside were significantly lower in fruit of red raspberry grown under high tunnels compared with those grown in an open field. Tunnel production may explain the low detection of pelargonidin-3-rutinoside in our study. The better fruit quality of tunnel-grown fruit, combined with targeted breeding for enhancement of health-beneficial compounds, could mitigate tunnel effects on reduced flavonoid content. More research is needed to determine how high-tunnel cultivation affects flavonoid content among red raspberry cultivars.

When evaluating the health benefits of raspberry phytochemicals in laboratory or clinical trials, or when breeding for enhanced antioxidant or phenolic levels, one must have an understanding of variation caused by genotype and environment. Certain cultivars and locations may be more amicable to the accumulation or enhancement of phytochemicals than others,[4] and these differences must be accounted for in both health research and food processing. Temperature effects on flavonoid composition could ultimately alter the results of laboratory and clinical trials examining the health benefits of whole berries and extracts, along with affecting the potency of botanicals, dietary supplements, and processed food products.[1] By using targeted metabolite analysis and practical, well-characterized laboratory assays, both genetic and environmental effects on flavonoid composition in red raspberry have been identified. The results of this study suggest that growing raspberries in a warmer climate may promote the accumulation of health-beneficial flavonoid compounds and that temperature

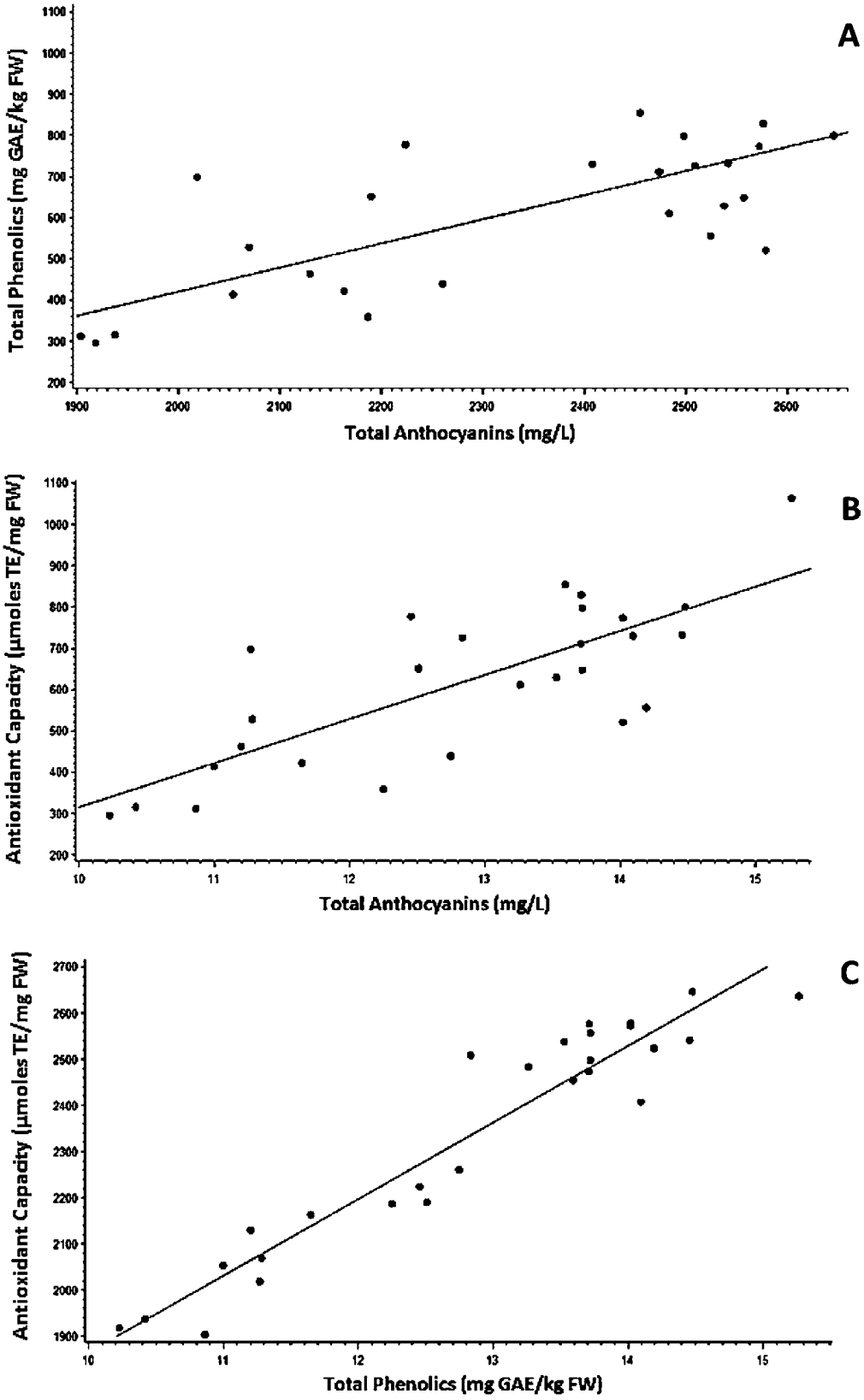

Figure 5. Correlation plot (A) between total anthocyanins and phenolics (R^2 = 0.544), (B) between total anthocyanins and antioxidant capacity (R^2 = 0.596), and (C) between total phenolics and antioxidant capacity (R^2 = 0.912). Each point on the graphs represents the average of three replicates, and values are displayed ± standard error.

effects must be accounted for in the breeding for enhanced phytochemical content.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Compounds that were detected by LC-TOF-MS but not quantified against their exact external standard are quantified against an appropriate external standard, and concentrations for all detected compounds are reported as (mg/g DW ± standard error). This information is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Ozgen, M.; Wyzgoski, F.; Tulio, A. Jr.; Gazula, A.; Miller, A.; Scheerens, J.; Reese, R.; Wright, S. Antioxidant capacity and phenolic antioxidants of midwestern black raspberries grown for direct markets are influenced by production site. *HortScience* **2008**, *43*, 2039−2047.

(2) Kassim, A.; Poette, J.; Paterson, A; Zait, D.; McCallum, S.; Woodhead, M.; Smith, K.; Hackett, C.; Graham, J. Environmental and seasonal influences on red raspberry anthocyanin antioxidant contents and identification of quantitative traits loci (QTL). *Mol. Nutr. Food Res.* **2009**, *53*, 625−634.

(3) Rao, A. V.; Snyder, D. M. Raspberries and human health: a review. *J. Agric. Food Chem.* **2010**, *58*, 3871−3883.

(4) Tokuşoğlu, Ö.; Stoner, G. Phytochemical bioactives in berries. In *Fruit and Cereal Bioactives*; Tokuşoğlu, Ö., Ed.; CRC Press: Boca Raton, FL, 2011; pp 143−162.

(5) Battino, M.; Beekwilder, J.; Donoyes-Rothan, B.; Laimer, M.; McDougall, G.; Mezzetti, B. Bioactive compounds in berries relevant to human health. *Nutr. Rev.* **2009**, *67*, S145−S150.

(6) Wang, S. Y.; Chen, C.; Wang, C. Y. The influence of light and maturity on fruit quality and flavonoid content of red raspberries. *Food Chem.* **2009**, *112*, 676−684.

(7) Deighton, N.; Brennan, R.; Finn, C; Davies, H. V. Antioxidant properties of domesticated and wild *Rubus* species. *J. Sci. Food Agric.* **2000**, *80*, 1307−1313.

(8) Heinonen, M. Antioxidant activity and antimicrobial effect of berry phenolics − a Finnish perspective. *Mol. Nutr. Food Res.* **2007**, *51*, 684−691.

(9) Seeram, N.; Adams, L.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* **2006**, *54*, 9329−9339.

(10) Fukusaki, E.; Kobayashi, A. Plant metabolomics: potential for practical operation. *J. Biosci. Bioeng.* **2005**, *100*, 347−354.

(11) Fiehn, O. Metabolomics − the link between genotypes and phenotypes. *Plant Mol. Biol.* **2002**, *48*, 155−171.

(12) Fernie, A. R.; Schauer, N. Metabolomics-assisted breeding: a viable option for crop improvement? *Trends Genet.* **2008**, *25*, 39−48.

(13) Mullen, W.; McGinn, J.; Lean, M.; MacLean, M.; Gardner, P.; Duthie, G.; Yokota, T.; Crozier, A. Ellagitannins, flavonoids, and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. *J. Agric. Food Chem.* **2002**, *50*, 5191−5196.

(14) Harrigan, G.; Martino-Catt, S.; Glenn, K. Metabolomics, metabolic diversity and genetic variation in crops. *Metabolomics* **2007**, *3*, 259−272.

(15) Freeman, B.; Stocks, J. C.; Eggett, D. L.; Parker, T. L. Antioxidant and phenolic changes across one harvest season and two storage conditions in primocane raspberries (*Rubus idaeus* L.) grown in a hot, dry climate. *HortScience* **2011**, *46*, 236−239.

(16) Anttonen, M. J.; Karjalainen, R. O. Environmental and genetic variation of phenolic compounds in red raspberry. *J. Food Compos. Anal.* **2005**, *18*, 759−769.

(17) Gonzalez, E. M.; deAncos, B.; Cano, M. P. Relation between bioactive compounds and free radical-scavenging capacity in berry fruits during frozen storage. *J. Sci. Food Agric.* **2003**, *83*, 722−726.

(18) Stewart, D.; McDougall, J. G.; Sungurtas, J.; Verrall, S.; Graham, J.; Martinussen, I. Metabolomic approach to identifying bioactive compounds in berries: advances toward fruit nutritional enhancement. *Mol. Nutr. Food Res.* **2007**, *51*, 645−651.

(19) Kafkas, E.; Ozgen, M.; Ozogul, Y.; Turemis, N. Phytochemical and fatty acid profile of selected red raspberry cultivars: a comparative study. *J. Food Qual.* **2008**, *31*, 67−78.

(20) Maatta-Riihinen, K.; Kamal-Eldin, A.; Torronen, A. Identification and quantification of phenolic compounds in berries of *Fragaria* and *Rubus* species (family Rosaceae). *J. Agric. Food Chem.* **2004**, *52*, 6178−6187.

(21) Remberg, S.; Sønsteby, A.; Aaby, K.; Heide, O. Influence of postflowering temperature on fruit size and chemical composition of Glen Ample raspberry (*Rubus idaeus* L.). *J. Agric. Food Chem.* **2010**, *58*, 9120−9128.

(22) Fernandez, G.; Louws, F.; Ballington, J. R.; Poling, E. B. *Growing Raspberries in North Carolina*; North Carolina Cooperative Extension Service Publication AG-569; North Carolina State University: Raleigh, NC, 1998.

(23) Jennings, D. L. *Raspberries and Blackberries: Their Breeding, Diseases, and Growth*; Academic Press: San Diego, CA, 1988.

(24) Giusti, M. M.; Wrolstad, R. E. Characterization and measurement of anthocyanins by UV-visible spectroscopy. *Curr. Protoc. Food Anal. Chem.* **2001**, F1.2.1−F1.2.13.

(25) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.

(26) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70−76.

(27) de Ancos, B.; Gonzalez, E.; Cano, M. P. Differentiation of raspberry varieties according to anthocyanin composition. *Z. Lebensm. Unters. Forsch. A* **1999**, *208*, 33−38.

(28) Ballington, J. R.; Fernandez, G. E.; Bryson, S. K. Raspberry named 'Nantahala'. USPP 20,689, Jan 26, 2010.

(29) Fernandez, G. E.; Ballington, J. R.; Bryson, S. K. 'Nantahala' red raspberry. *HortScience* **2009**, *44*, 25−26.

(30) Sanford, J.; Ourecky, D. 'Royalty' − a purple-red raspberry. *N. Y. Food Life Sci. Bull.* **1982**, 97.

(31) Jennings, D. L.; Carmichael, E. Anthocyanin variation in the genus *Rubus*. *New Phytol.* **1980**, *84*, 505−513.

(32) Mullen, W.; Lean, M.; Crozier, A. Rapid characterization of anthocyanins in red raspberry fruit by high-performance liquid chromatography coupled to single quadrupole mass spectrometry. *J. Chromatogr., A* **2002**, *966*, 63−70.

(33) Demchak, K. Small fruit production in high tunnels. *HortTechnology* **2009**, *19*, 44−49.

(34) Heidenreich, C.; Pritts, M.; Kelly, M.; Demchak, K. *High Tunnel Raspberries and Blackberries*; Cornell University Department of Horticulture: Ithaca, NY, 2008; Vol. *47*.